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TECHNICAL MANUSCRIPT 431

SPECIFIC IDENTIFICATION OF FRACTION-I-POSITIVE
PASTEURELLA PESTIS COLONIES ON ANTISERUM-AGAR PLATES

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In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Jerry D. Gordon. We thank Earl D. Beesley for helpful suggestions and for providing characterized laboratory stock bacterial strains. We are grateful to Dr. William D. Lawton for providing a Fraction I antigen-antibody system for gel diffusion tests.

ABSTRACT

A method is described for using antiplague serum in blood agar base plating media to detect Fraction-I-positive Pasteurella pestis. The antiserum was produced conveniently and in large volume in rabbits utilizing Cutter plague vaccine combined with Freund's complete adjuvant. P. pestis colonies were specifically identified within 48 hours after plating by the presence of a precipitin ring surrounding each colony. The basis of the test was shown to be a precipitin reaction between Fraction I antigen released from P. pestis colonies after chloroform vapor treatment and Fraction I antibody present in the antiserum-agar medium.

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I. INTRODUCTION

Serological techniques currently used for detecting or identifying Pasteurella pestis are based upon the specific reaction between Fraction I antigen produced by plague organisms at 37 C and specific antibody produced against Fraction I antigen.¹⁻⁷ These methods detect Fraction I antigen present in cultures, culture extracts, or tissues and require either specific Fraction I antibody or purified Fraction I antigen and, in some cases, prior isolation of the suspected organism.

This paper describes an additional serological test for P. pestis that utilizes the specific Fraction I antigen-antibody reaction. By this method, P. pestis colonies originating largely from single cells can be identified on antiserum-agar plates. A large supply of specific antiserum, required for extensive use of the method, was conveniently obtained using commercially available vaccine and adjuvant. The nature of the test allows for the feasibility of identifying P. pestis colonies in the presence of contaminating organisms.

II. MATERIALS AND METHODS

A. PRODUCTION OF ANTISERA

Each of four New Zealand White rabbits weighing 2.5 to 3 kg was injected initially with 2 ml of an equal mixture of Cutter plague vaccine* and Freund's complete adjuvant.** This dose (containing 2×10^9 organisms) was divided into 0.75 ml injected into the large muscle of each hind leg and 0.5 ml injected subcutaneously in the dorsal neck region. One week later, each rabbit was injected with 1 ml of undiluted Cutter plague vaccine (2×10^9 organisms without adjuvant), 0.5 ml intramuscularly into one hind leg and 0.5 ml subcutaneously in the dorsal neck region. Four uninoculated rabbits served as a source of normal rabbit serum throughout the experiment. Rabbits were bled from the marginal ear vein before receiving the first injection, again after the 6th week, and weekly thereafter through the 16th week. Xylene was applied locally to the rabbit's ear to facilitate collection of 40 ml of blood from each rabbit per bleeding. Antisera were sterilized by filtering through 0.45-micron disposable Nalgene filter units*** under only slight vacuum.

* Cutter Laboratories, Berkeley, California.

** Difco Laboratories, Detroit, Michigan.

*** Nalge Company, Inc., Rochester, New York.

B. SCHEDULE FOR TESTING ANTISERA

Sera obtained from normal and immunized rabbits each week were tested individually for Fraction I antibody titer using the gel diffusion technique of Ouchterlony⁸ and the bacterial agglutination test employing Cutter plague vaccine as a source of Fraction I antigen. Individual antisera were combined in equal volumes to form weekly pools of antisera that were, in turn, tested for Fraction I antibody titer. Weekly antiserum pools and a large antiserum pool containing equal volumes of the antisera collected from the 9th through the 16th week were tested for precipitin ring titer and specificity of reaction in antiserum-agar plates.

C. GEL PRECIPITIN TEST

Standard disposable petri plates (100 x 15 mm) containing a mixture of 1% ion agar No. 2,* 0.01% merthiolate, and 0.9% NaCl were used to determine gel precipitin titers. Seven wells 0.5 mm in diameter and 2 mm apart were punched into the solidified diffusion agar using a hollow cylindrical steel borer and a plastic template. Cutter plague vaccine (undiluted) was deposited in the central well. After standard Fraction I antiserum⁹ was deposited in one of the outer wells, twofold dilutions of antisera in 0.85% NaCl were deposited in four of the five remaining wells. One well located between the Fraction I antibody standard and the last dilution of the test serum was left vacant to avoid false end points. Parallel tests were conducted using normal rabbit sera collected at the same time and processed identically to the antiplague sera. Gel diffusion plates were incubated 18 to 24 hours at 26 C and observed through a dissecting microscope using oblique transmitted light. The last dilution of antiserum producing a distinct precipitin band continuous with the precipitin band formed between the Fraction I antigen source and the Fraction I antibody standard was considered the end point of the titration.

D. BACTERIAL AGGLOUTINATION TEST

A volume of 0.1 ml of Cutter plague vaccine (freshly diluted 1:10 in 0.85% NaCl) was mixed in the small depressions of a disposable agglutination tray with an equal volume of antiserum diluted in 0.85% NaCl. Parallel tests were conducted using normal rabbit sera collected at the same time and processed identically to the antiplague sera. After mixing the components by a gentle circular motion of the tray, a sheet of plastic wrap was placed over the entire tray, and the tray was incubated at 26 C. After incubation for 18 to 24 hours, agglutination trays were viewed through a dissecting microscope with oblique transmitted light. The last dilution of antiserum producing pronounced, large flaky aggregates was considered the end point of the titration.

* Consolidated Laboratories, Inc., Chicago Heights, Illinois.

E. PREPARATION OF ANTISERUM-AGAR PLATES

Filtered antiserum was diluted with sterile 0.85% NaCl, brought to 37 C in a water bath, and added to an equal volume of sterile 8% blood agar base (Difco) at pH 6.8 that had been allowed to cool for at least 1 hour in a water bath maintained at 55 C. The antiserum-agar mixture was swirled gently in a flask to mix the components completely without the excessive formation of air bubbles. Rapidly, 8 to 10 ml of antiserum-agar were poured into sterile, standard-size disposable petri plates. Uniform plates were prepared by covering one-half of the bottom surface of each petri plate with antiserum-agar and immediately tilting the plate to distribute the material over the entire bottom surface of each plate. Plates were sealed in plastic bags immediately after the agar solidified and were stored at 4 C until used.

F. BACTERIAL TEST STRAINS

Bacterial strains were standard laboratory stocks with the exception of clinical isolates: Streptococcus pyogenes (group A),* Streptococcus viridans,* and Staphylococcus aureus (80-81 phage type).**

G. PRECIPITIN RING TEST IN ANTISERUM-AGAR PLATES

Bacterial test strains grown on Difco blood agar base slants for 18 to 24 hours at 26 C were diluted 7 logs with sterile 2.5% heart infusion broth (Difco). Of this dilution, 0.1 ml (containing 50 to 100 organisms) was spread on the surface of antiserum-agar plates using glass spreading rods. Pneumococcal and streptococcal strains were grown on blood agar slants for 48 hours at 26 C and diluted 6 logs for application to antiserum-agar plates. Plates were incubated at 37 C for 40 hours or until P. pestis colonies were 0.5 to 1 mm in diameter. Plates were then exposed to chloroform vapor for 10 minutes by inverting them over inverted glass petri dish covers containing 2 ml of chloroform on a gauze sponge (2 inches by 2 inches, 12 ply). The plates were then removed and left inverted (agar surface down) in a tilted position for 10 minutes to eliminate chloroform vapor. Chloroform-treated plates were incubated at 37 C and examined after 2, 4, 6, and 24 hours for precipitin ring formation. Observations were made with a dissecting microscope and oblique transmitted light.

* Obtained through the courtesy of Dr. Martha K. Ward.

** Obtained through the courtesy of Dr. Robert A. Altenbern.

H. COMPARISON OF PRECIPITIN RINGS WITH A STANDARD FRACTION I ANTIGEN-ANTIBODY SYSTEM

P. pestis strain Alexander was plated on antiserum-agar plates containing pooled (9 to 16 weeks) antiplague serum diluted 1:128. The plates were incubated for 48 hours and subsequently exposed to chloroform vapor for 10 minutes. Immediately, two wells were punched with a special 13-gauge needle in the nutrient agar adjacent to each of several P. pestis colonies. Standard Fraction I antigen and Fraction I antibody* were added to the wells adjacent to P. pestis colonies. The plates were incubated at 37 C and examined within 24 hours.

I. REPLICATION OF COLONIES

Fraction-I-positive P. pestis colonies grown on antiserum-agar plates for 48 hours at 37 C were replicated by use of velveteen to a second antiserum-agar plate containing pooled antiplague serum diluted 1:128. The original plate was then treated with chloroform vapor for 10 minutes. The replicate plate was incubated at 37 C for 48 hours before being subjected to chloroform vapor treatment for 10 minutes.

J. VARIATION OF CHLOROFORM VAPOR TREATMENT

Forty-hour P. pestis colonies (0.5 to 1 mm in diameter) grown at 37 C on antiserum-agar plates were exposed to chloroform vapor for 15 and 30 seconds, and 1, 1.5, 2, 2.5, and 5 minutes. Plates were incubated at 37 C for 24 hours and observed to determine the shortest exposure to chloroform vapor that would result in precipitin ring formation around all colonies present on each plate. Several colonies from each plate were picked and streaked onto plates containing blood agar base (Difco). Plates were incubated at 26 C for 48 hours and observed to determine the longest exposure to chloroform vapor that would permit viability of all colonies picked from each plate.

III. RESULTS

Gel precipitin and bacterial agglutinin titers of individual and pooled antisera were elevated by the 9th week and remained reasonably high through the 16th week (Table 1). The large pool of antiserum collected from the 9th through the 16th week exhibited a comparable antibody titer. Normal rabbit sera used in a pa allel study failed to exhibit antibody titers against the test antigen.

* Provided by Dr. William D. Lawton.

TABLE 1. WEEKLY GEL PRECIPITIN AND AGGLUTININ TITERS OF INDIVIDUAL AND POOLED RABBIT ANTIPLAGUE SERA TESTED AGAINST CUTTER PLAGUE VACCINE

Weeks Post Immunization	Reciprocal of Antibody Titer								Rabbits 1 to 4 Pooled	
	Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 4		P	A
	P ^a	A ^b	P	A	P	A	P	A		
0	0	0	0	0	0	0	0	0	0	0
6	128	16	64	16	32	8	16	8	32	8
7	128	16	128	32	32	8	32	8	32	8
8	128	32	128	32	32	8	32	8	64	16
9	128	32	128	32	64	8	64	16	128	32
10	256	64	64	32	128	16	64	64	128	64
11	256	32	128	32	128	16	128	16	128	32
12	256	32	128	32	256	16	128	16	256	32
13	256	64	256	32	256	16	128	16	256	32
14	256	64	256	64	128	32	128	16	256	32
15	256	32	256	32	128	32	128	16	256	32
16	256	32	128	32	128	16	64	16	256	16
9-16 (pool)									256	32

a. P = precipitin titer.

b. A = agglutinin titer.

Weekly antiserum pools collected during this study were tested for precipitin ring titer (Table 2) and specificity (Table 3) in antiserum-agar plates. Precipitin ring titers (Table 2) were elevated by the 7th week, reached a peak at the 13th week and remained elevated through the 16th week. The large antiserum pool (9 to 16 weeks) showed a precipitin ring titer equal to the highest weekly titers obtained during the experiment. Normal rabbit sera failed to react with the test antigen in a parallel study.

TABLE 2. WEEKLY PRECIPITIN RING TITERS OF POOLED RABBIT ANTIPLAGUE SERA
AGAINST P. PESTIS COLONIES (STRAIN ALEXANDER)
IN ANTISERUM-AGAR PLATES

Weeks Post Immunization	Formation of Precipitin Rings at Indicated Reciprocal of Final Serum Dilution					
	16	32	64	128	256	512
0	-	-	-	-		
6	+	+	+	-		
7	+	+	+	+	-	
8	+	+	+	+	-	
9		+	+	+	-	
10		+	+	+	-	
11		+	+	+	-	
12		+	+	+	-	
13		+	+	+	+	+
14		+	+	+	+	+
15		+	+	+	+	+
16		+	+	+	+	+
9-16 (pool)		+	+	+	+	+

TABLE 3. WEEKLY TESTS OF SPECIFICITY OF POOLED RABBIT ANTIPLAGUE SERA
FOR FRACTION-I-POSITIVE COLONIES IN ANTISERUM-AGAR PLATES

Weeks Post Immunization	Precipitin Ring Formation at Final Serum Dilution 1:32			
	<u>P. pestis</u> Alexander	<u>P. pestis</u> M23	<u>P. pstb.</u> PB1 ⁺	<u>E. coli</u> Type B
0	-	-	-	-
6	+	-	-	-
7	+	-	-	-
8	+	-	-	-
9	+	-	-	-
10	+	-	-	-
11	+	-	-	-
12	+	-	-	-
13	+	-	-	-
14	+	-	-	-
15	+	-	-	-
16	+	-	-	-
9-16 (pool)	+	-	-	-

All weekly antiserum pools and the large pool of antiserum collected from the 9th through the 16th week were specific for Fraction-I-positive P. pestis colonies (strain Alexander) when tested at the 1:32 dilution (Table 3). Corresponding normal rabbit serum pools did not form precipitin rings with P. pestis colonies (strain Alexander).

As indicated in Table 2, the large antiserum pool (9 to 16 weeks) showed a precipitin ring titer of 1:512 in antiserum-agar plates. P. pestis colonies from this titration are shown in Figure 1. The precipitin ring appearing at the 1:16 dilution of the antiserum (A) represents antibody other than Fraction I antibody. This antibody is effectively diluted out at the 1:32 dilution, while Fraction I antibody becomes sufficiently dilute to permit a precipitin ring to form close to the P. pestis colony (B). Optimal precipitin rings were observed at the 1:128 dilution (D); therefore, this dilution of the large antiserum pool was selected as the standard dilution for use in antiserum-agar plates.

Several well-characterized strains of P. pestis (including some Fraction-I-negative strains), Pasteurella pseudotuberculosis, and strains from other genera were tested in antiserum-agar plates containing pooled antiserum diluted 1:128 (Table 4). Only those strains of P. pestis known to produce Fraction I promoted precipitin ring formation. Colonies from four of the several bacterial strains used in the specificity test are shown in Figure 2. Of the two P. pestis strains shown in Figure 2, only Alexander (A) produces Fraction I antigen.

P. pestis Alexander (a Fraction-I-positive strain) did not form precipitin rings if colonies were allowed to develop at 26 C. Likewise, P. pestis Alexander colonies did not form precipitin rings if allowed to grow 4 days at 37 C in the absence of chloroform vapor treatment. Negative results were also obtained when P. pestis colonies (Alexander) were grown at 37 C on nutrient agar containing normal rabbit serum diluted 1:128. These results are presented in Figure 3.

Chloroform vapor treatment for 10 minutes initially used to hasten the formation of precipitin rings killed the organisms. To facilitate further study of P. pestis, colonies were replicated to another antiserum-agar plate prior to treating the original plate with chloroform vapor. Figure 4 shows both original and replicate plates after they were treated with chloroform vapor for 10 minutes. Colony patterns were similar on both plates, demonstrating the validity of using a replication procedure in conjunction with the antiserum-agar method.

To permit further study of P. pestis colonies without resorting to replication procedures, antiserum-agar plates containing P. pestis colonies were exposed briefly to chloroform vapor. Results indicated that 30 seconds' exposure to chloroform vapor was sufficient to produce precipitin rings in all of the Fraction-I-positive P. pestis strains shown in Table 4. The test was specific for Fraction-I-positive P. pestis, using a chloroform exposure time of 30 seconds, because Fraction-I-negative P. pestis, P. pseudotuberculosis, and strains from other genera were unreactive. Improved precipitin rings were obtained with chloroform exposure times of 1 and 1.5 minutes without loss of specificity or viability of the colonies. Some colonies were killed after a 2-minute exposure to chloroform vapor, and all of the colonies were killed after 2.5 minutes.

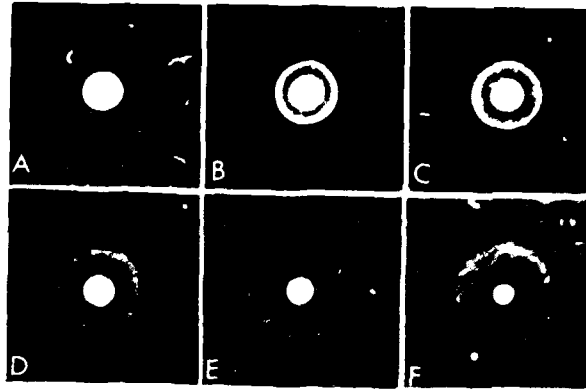


Figure 1. Precipitin Rings Surrounding *P. pestis* Colonies Grown on Nutrient Agar Containing Anti plague Serum (9 to 16 Week Pool). Dilutions: A, 1:16; B, 1:32; C, 1:64; D, 1:128; E, 1:256; and F, 1:512.

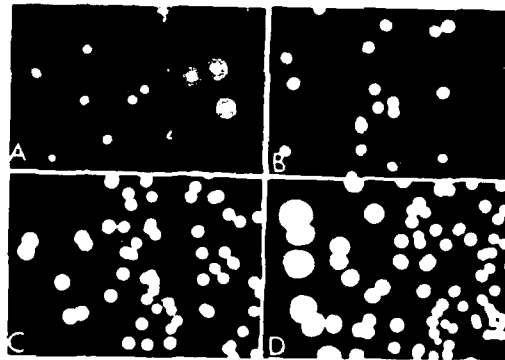


Figure 2. Specificity of Pooled Rabbit Anti plague Serum for Fraction-I-Positive *P. pestis* Colonies (Alexander) in Antiserum-Agar Plates. A, *P. pestis* (Alexander); B, *P. pestis* (M23); C, *P. pseudotuberculosis* (PBI⁺); and D, *E. coli* (Type B).

TABLE 4. SPECIFICITY OF POOLED (9 TO 16 WEEKS) BARRT ANTIPLAQUE SERUM (1:128 DILUTION) FOR FRACTION-I-POSITIVE P. PESTIS COLONIES IN ANTISERUM-AGAR PLATES

<u>P. pestis</u> (PI ⁺)a/ Ring	Pptn Ring	<u>P. pestis</u> (PI ⁻)b/ Ring	Pptn Ring	Other Genera	Pptn Ring
Alexander	+	M23	-	<u>Streptococcus pyogenes</u> (group A)	-
Kim 10	+	019	-	<u>Streptococcus viridans</u> (human throat)	-
Salizar	+	B14	-	<u>Staphylococcus aureus</u> (80-81 phage type)	-
Shasta	+	G35	-	<u>Diplococcus pneumoniae</u> (type I)	-
Saka	+	Jaffa	-	<u>Klebsiella pneumoniae</u> ATCC 13883	-
Yokohama	+	Java	-	<u>Salmonella typhosa</u> ATCC 167	-
MP6	+	TRU	-	<u>Shigella sonnei</u> ATCC 9290	-
P477	+	<u>P. patb</u>	-	<u>Escherichia coli</u> B	-
Bryans (M41)	+	PB1 ⁺ (I)c/	-	<u>Pasteurella X</u> (Winblad)	-
A4	+	EP2 ⁺ (II)	-	<u>Yersinia enterocolytica</u>	-
A12	+	MD31(III)	-	<u>Proteus morganii</u> ATCC 8019	-
A16	+	Neilson(IV)	-	<u>Enterobacter aerogenes</u> ATCC 13048	-
G32	+	PBS(25) (V)	-	<u>Pseudomonas aeruginosa</u> ATCC 10145	-
EV76	+	Alaska(1B)	-	<u>Serratia marcescens</u> ATCC 13880	-

a. Fraction I positive strains.

b. Fraction I negative strains.

c. Roman numerals indicate serotypes of P. pseudotuberculosis.

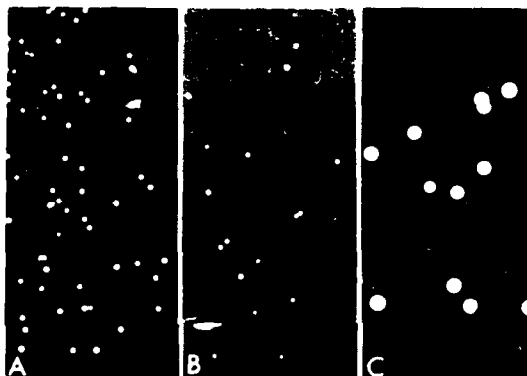


Figure 3. Negative Reactions Observed with P. pestis (Alexander) in Antiserum-Agar and Normal Serum-Agar Plates. A, Antiserum-Agar (26 C); B, normal serum-agar (37 C); and C, antiserum-agar (37 C) 4 days without chloroform.

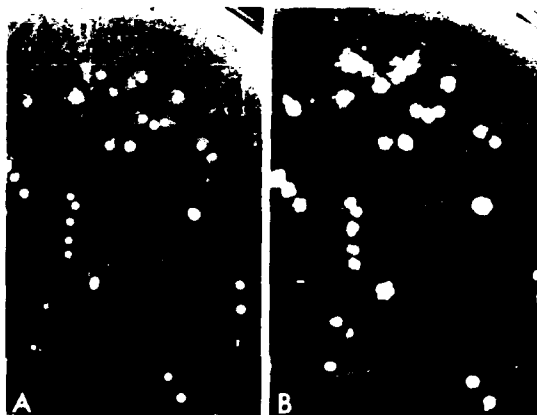


Figure 4. Replication of P. pestis Colonies (Alexander) from Antiserum-Agar Plate. A, Original plate; B, replicate plate.

Evidence gained during this study indicated that precipitin ring formation was based upon the specific reaction between Fraction I antigen released from P. pestis cells after chloroform vapor treatment and Fraction I antibody present in nutrient agar plates. Direct evidence of this is presented in Figure 5. The joining of the precipitin rings surrounding both antigen sources and the dissolving of the precipitin ring between antigen sources A and B are considered evidence that the two precipitin reactions are homologous.

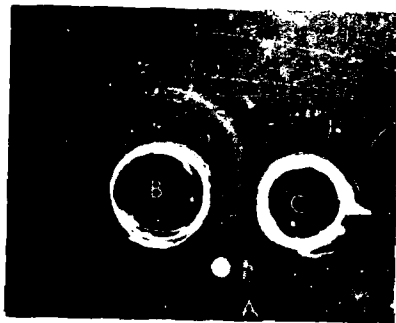


Figure 5. Comparison of Precipitin Ring Surrounding Fraction-I-Positive P. pestis Colony with Standard Fraction I Antigen-Antibody System. A, P. pestis colony; B, Fraction I antigen; and C, Fraction I antibody.

IV. DISCUSSION

The diagnostic potential of identifying bacterial colonies growing on nutrient agar containing specific antiserum by the formation of precipitin rings surrounding the colonies was demonstrated many years ago;¹⁰⁻¹² however, until the present time, this concept has not been successfully applied to P. pestis.

Delay in developing an antiserum-agar method for P. pestis may be attributed to two major factors: (i) the difficulty involved in obtaining large supplies of specific Fraction I antisera and (ii) the tendency for P. pestis to hold Fraction I antigen tightly when grown on agar surfaces.

Both obstacles were overcome in the present study because a large volume of specific antiplague serum was obtained without first resorting to the task of purifying Fraction I antigen,¹³ and chloroform vapor was utilized to release Fraction I antigen from P. pestis cells. The method of antiserum production reported in this study was selected on the basis of the following considerations: (i) a large supply of specific antiserum would be required for extensive use of the antiserum-agar method, (ii) the method of antiserum production should be convenient and should utilize a standard antigen to minimize variables, (iii) animals injected with Cutter plague vaccine and Freund's adjuvant yielded antisera containing Fraction I antibody and only rarely other detectable antibodies,⁹ (iv) studies by Chen, Larson, and Meyer¹⁴ showed that formalin-killed P. pestis evoked highest antibody response when given at high antigenic mass with aluminum hydroxide adjuvant, (v) Freund's complete adjuvant is a potent adjuvant of long standing and can be obtained commercially (Gifco) to minimize variables, (vi) our preliminary experiments with groups of rabbits immunized with Cutter plague vaccine and Freund's complete adjuvant using different schedules and dosages indicated that Fraction I antibody titers remained elevated over a period of several weeks and that two massive injections spaced 1 week apart consistently gave higher titers, and (vii) combining all considerations, it was assumed that injection into rabbits of a large antigenic mass of Cutter plague vaccine with Freund's complete adjuvant would result in the production of antiserum that would contain predominantly Fraction I antibody over a period of several weeks. The antiserum could then be made specific for Fraction-I-positive P. pestis simply by diluting out the unwanted antibodies.

It is not clear why Fraction I antibody should predominate in rabbits injected with a massive dose of Cutter plague vaccine and Freund's complete adjuvant. Perhaps formalin used to kill the cells during vaccine production and/or phenol used as a preservative preferentially alter or damage antigens other than Fraction I. Perhaps formalin alters Fraction I, making it a more effective antigen (in rabbits) in combination with adjuvant, as Chen, Larson, and Meyer¹⁴ have concluded is true in the

case of guinea pigs. The antiplague serum produced for use in the present method is not pure in the absolute sense. Antibody other than Fraction I antibody may be present at concentrations too dilute to permit visible reaction. Conceivably, antibody other than Fraction I antibody could be present at concentrations sufficiently high to permit reaction; however, the reaction may not occur because the corresponding antigen may not be produced in colonies grown on agar surfaces. On the other hand, it may be that the corresponding antigen is produced but is obscured by Fraction I antigen and is therefore not available for diffusion or release by chloroform vapor treatment. If the latter were true, one could perhaps liberate the antigen with solvents other than chloroform or a combination of solvents used simultaneously or in series. Whatever the actual situation with regard to additional antibodies in the antiserum and the presence or absence or availability of other antigens within the cells of the colonies, it may be stated that, under the conditions of the present antiserum-agar test, the antiserum is specific for Fraction-I-positive P. pestis.

The antiserum-agar technique, based on precipitin rings surrounding colonies grown on nutrient agar containing specific antiserum, was investigated because the concept offered possibilities for pursuing areas of plague research not attainable with current methods. The method is especially suited to P. pestis because fully virulent P. pestis strains¹⁵ and most known avirulent and attenuated strains produce Fraction I antigen. Also, it is advantageous that Fraction I antigen is unique to P. pestis. Even P. pseudotuberculosis, the closest known antigenically related relative of P. pestis, does not produce Fraction I antigen. The effectiveness of the antiserum-agar method is vividly demonstrated in the two following observations. The Alaskan isolate of P. pseudotuberculosis that yielded a false positive immunofluorescent reaction with Fraction Ib antibody¹⁶ failed to react in the present test system. At the other extreme, the Bryans isolate of P. pestis that yielded a negative reaction in fluorescent staining tests employing specific Fraction I antibody¹⁷ was readily detected using our antiserum-agar technique.

The antiserum agar method for P. pestis was designed with certain ultimate expectations. With the antiserum-agar method employing Fraction I antibody, one should be able to determine the Fraction I (genetic potential) of individual cells within a P. pestis population, assuming of course that the colonies are derived largely from single cells. This would be most helpful to anyone concerned with mutations or genetic exchange involving Fraction I antigen. Fraction-I-positive P. pestis also should be detectable in the presence of contaminating organisms by using selective media containing specific Fraction I antibody. Simultaneous isolation and identification of P. pestis could be accomplished on antiserum-agar plates, thus simplifying procedures for confirming the clinical diagnosis of plague and identifying P. pestis in ecological surveys.

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13. ABSTRACT A method is described for using antiplague serum in blood agar base plating media to detect Fraction-I-positive <u>Pasteurella pestis</u> . The antiserum was produced conveniently and in large volume in rabbits utilizing Cutter plague vaccine combined with Freund's complete adjuvant. <u>P. pestis</u> colonies were specifically identified within 48 hours after plating by the presence of a precipitin ring surrounding each colony. The basis of the test was shown to be a precipitin reaction between Fraction I antigen released from <u>P. pestis</u> colonies after chloroform vapor treatment and Fraction I antibody present in the antiserum-agar medium.		
14. Key Words * <u>Pasteurella pestis</u> *Antiserum-agar *Identification Fraction I Antibody Plague		

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